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Identification of D^b- and K^b-Restricted Subdominant Cytotoxic T-Cell Responses in Lymphocytic Choriomeningitis Virus-Infected Mice

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Antiviral cytotoxic T-cells are critical for control of lymphoctytic choriomeningitis virus (LCMV) infection in mice. In H-2^b mice, the antiviral response is directed against three D^b-restricted epitopes in the viral nucleoprotein (NP396-404) and glycoprotein (GP276-286 and GP33-41). Our present data revealed a clear hierarchy among these three epitopes, in which NP396-404 is immunodominant, followed by GP33-41 and GP276-286, respectively. In order to identify additional CTL epitopes in the LCMV nucleoprotein and glycoprotein, we used the motifs for D^b- and K^b-binding peptides, combined with MHC class I-binding assays. Out of 23 D^b motif-fitting peptides, we identified 4 D^b binders, one of which (GP92-101) turned out to be a new CTL epitope. Among 28 K^b motif-fitting peptides, 12 bound K^b, and one of these (NP205-212) was a CTL epitope. Both newly identified CTL peptides were recognized by LCMV-immune splenocytes after secondary *in vitro* stimulation. Both peptides bound their MHC class I molecules with intermediate affinity (470 and 170 nM for GP92-101 and NP205-212, respectively). Responses against these peptides were weaker than the responses against the three major epitopes. None of the high affinity binders were new epitopes, suggesting that high affinity binders are either immunodominant epiitopes or no epitopes at all. Thus, analysis of 51 K^b and D^b motif-fitting peptides yielded 2 new, subdominant epitopes. Immunization of C57BL/6 mice with these peptides, or with vaccinia virus recombinants expressing these epitopes as minigenes, protected against chronic LCMV infection, demonstrating that immunization with subdominant epitopes can confer protection against chronic viral infection. © 1998 Academic Press

INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) induces a potent antiviral immune response in its murine natural host. Antiviral $CD8^+$ cytotoxic T lymphocytes (CTLs) are crucial for control of the viral infection. Through their T-cell receptors (TCR), CTLs recognize small antigenic peptides that are associated with major histocompatability complex (MHC) class I molecules (Schumacher *et al.,* 1991; Townsend and Bodmer, 1989; Van Bleek and Nathenson, 1990). In the case of LCMV infection in mice of the H-2^b haplotype (e.g., C57BL/6 mice), the antiviral response is largely directed against three dominant epitopes, i.e., residues 396–404 from the nucleocapsid protein (FQPQNGQFI), residues 33–41 from the glycoprotein (KAVYNFATC), and residues 276–286 from the glycoprotein (SGVENPGGYCL) (Gairin *et al.,* 1995; Klavinskis *et al.,* 1990; Oldstone *et al.,* 1988; Schulz *et al.,* 1989; Selin *et al.,* 1996). These three peptides are presented by the D^b MHC class I molecule and are therefore referred to as D^b-restricted.

In several different experimental systems, it has been shown that there is a hierarchy in the epitope specificity of MHC class I-restricted responses (Feltkamp *et al.,* 1995; Jameson and Bevan, 1992; Oukka *et al.,* 1994; Van der Most *et al.,* 1996; Vitiello *et al.,* 1996). CTL epitopes can be dominant, subdominant, or cryptic. Several different factors can contribute to the dominance or subdominance of a potential epitope, such as (i) the intracellular processing of the antigenic peptide, (ii) its MHC class I-binding affinity, and (iii) the TCR repertoire. In the present study, we have investigated the existence of LCMV-specific, H-2^b-restricted subdominant responses. Eventually, identification of subdominant epitopes could be a first step toward the development of therapeutic vaccination protocols, i.e., immunization to combat an established infection.

A proven strategy to identify new CTL epitopes, is to use allele-specific sequence motifs for MHC class I-binding peptides, combined with MHC-binding assays (Kast *et al.,* 1994; Van der Most *et al.,* 1996; Vitiello *et al.,* 1996). Allele-specific epitope motifs, first discovered after sequence analysis of pools of naturally processed peptides eluted from class I molecules, have now been

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identified for several human as well as murine class I alleles. They usually consist of two or more conserved anchor residues and comprise between 8 and 11 amino acids (Rammensee *et al.,* 1995). We have used the motifs described for the D^b and K^b class I alleles to search for new LCMV CTL epitopes. Motif-fitting peptides were synthesized and tested for MHC class I binding using recently developed D^b- and K^b-binding assays (Vitiello *et al.,* 1996). This approach resulted in the identification of four D^b-binding peptides and twelve K^b binders. Among these peptides, two were found to be new, subdominant epitopes; both peptides are recognized by antiviral CTL after secondary stimulation and were shown to confer protective immunity against chronic viral infection.

MATERIALS AND METHODS

Mice

Five- to eight-week-old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred at our colony at the University of California at Los Angeles.

Virus

LCMV strain Armstrong CA1371 (Ahmed *et al.,* 1984) and its derivative clone 13 (Ahmed *et al.,* 1991, 1984; King *et al.,* 1990; Matloubian *et al.,* 1993) were used in this study. LCMV clone 13 was isolated from the spleens of BALB/c LCMV carrier mice infected at birth with Armstrong. LCMV-immune mice were obtained by infecting mice ip with 2×10^5 PFU of LCMV Armstrong. Immune mice were used in experiments at >30 days post infection (p.i.). For challenge experiments, mice were infected iv with 1.5×10^6 PFU of LCMV clone 13.

Cell lines

MC57 cells (H-2^b) were used as targets in cytotoxicity assays. The mouse lymphoma line EL-4 was used as a source of D^b and K^b class I molecules. MC57 cells were used as target cells rather than EL-4 cells, since MC57 cells gave consistently better results, i.e., higher levels of ^{51}Cr release than EL-4, using the same effector cells. MHC I expression levels in MC57 cells were similar, if not higher, than in EL-4 cells. Vero cells were used for virus titration. MC57 and EL-4 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics. Vero cells were maintained in EMEM supplemented with 10% FCS, 2mM L-glutamine, and antibiotics. Large quantities of EL-4 cells were grown in spinner cultures.

Affinity purification of H-2^b molecules and MHC Class I peptide-binding assay

H-2^b molecules were purified from EL-4 cell lysates by affinity chromatography, as described previously (Sette *et al.,* 1994; Vitiello *et al.,* 1996) using monoclonal antibodies Y3 (anti H-$2K^b$) or 28-14-8s (anti H-$2D^b$). H-$2D^b$ and H-$2K^b$ peptide-binding assays were done as previously described (Sette *et al.,* 1989; Vitiello *et al.,* 1996). The radiolabeled probes utilized, and their average IC50s in the respective assays, were a $P_7 \rightarrow Y$ analog (SGPSNTYPEI, 4.4 nM) of the adenovirus E1A epitope (Kast *et al.,* 1989) for D^b and the VSV NP52-59 epitope (RGYVFQGL, 3.1 nM) (Van Bleek and Nathenson, 1990) for K^b.

Peptide synthesis

Peptides were either synthesized at Cytel as previously described (Ruppert *et al.,* 1993) or, for large epitope libraries, purchased as crude material from Chiron Mimotopes (Chiron Corp., San Diego, CA). Peptides synthesized at Cytel were purified to >95% homogeneity by reverse-phase HPLC. The purity of the synthetic peptides was assayed on an analytical reverse-phase column, and their composition was ascertained by amino acid analysis, sequencing, and/or mass spectrometry analysis.

Peptide immunization

Lipidated peptides, comprising a CTL epitope covalently attached to a T-helper epitope (chicken ovalbumin 323-336, ISQAVHAAHAEINE) and a lipid moiety (Vitiello *et al.,* 1995), were dissolved in PBS (10 nmol in 100 μl PBS) and injected sc at the base of the tail. Mice were challenged with 1.5×10^6 PFU LCMV clone 13 at least 15 days after immunization.

Construction of vaccinia viruses encoding LCMV CTL epitopes

Oligonucleotides encoding the relevant epitopes, preceded by an ATG start codon, and with a termination codon immediately following the open-reading frame, were synthesized (Gibco BRL), and were cloned into a vaccinia transfer plasmid downstream of the p7.5 vaccinia promoter. Using these transfer plasmids, vaccinia recombinants were generated by standard procedures (Mackett *et al.,* 1984; Whitton *et al.,* 1988), and were subjected to four rounds of plaque purification. Virus stocks were grown in HeLa cells. The DNA sequence of each insert was confirmed by PCR sequencing of the recombinant virus DNA as previously described (Sheng *et al.,* 1993). The epitope sequences, including the methionine initiator, are MYTVKYPNL (NP205-212), MCSANNSHHYI (GP92-101), and MGYCLTKWMI (GP283-291). The latter sequence, encompassing a K^d-restricted LCMV epitope (Van der Most *et al.,* 1997, 1996) was used to construct the control vaccinia virus recombinant.

TABLE 1

D^b Binding of LCMV Peptides

Peptide[a]	Sequence[a]	Binding capacity IC_{50} (nM)[b]
NP396-404	FQPQNGQFI	4.4
GP276-286	SGVENPGGYCL	52
GP33-41	KAVYNFATC	5429
GP33-43	KAVYNFATCGI	776
GP392-400	WLVTNGSYL	128
GP92-101	CSANNSHHYI	470
GP110-118	LTFTNDSII	1462
NP550-558	FRGPNVVTL	3455
NP166-175	SLLNNQFGTM	6333
NP207-215	VKYPNLNDL	>10000
NP266-274	IKPSNSEDL	>10000
NP45-53	SEVSNVQRI	>10000
NP464-472	LDSQNRKDI	>10000
NP83-91	TSKKNVLKV	>10000
NP45-54	SEVSNVQRIM	>10000
NP255-264	LDGGNMLESI	>10000
NP293-302	VGDRNPYENI	>10000
NP372-381	GIDPNAPTWI	>10000
NP384-393	EGRFNDPVEI	>10000
GP117-125	IISHNFCNL	>10000
GP321-329	LIDYNKAAL	>10000
GP16-24	DEVINIVII	>10000
GP160-168	CDFNNGITI	>10000
GP150-158	RGNSNYKAV	>10000
GP411-420	QEADNMITEM	>10000
GP159-168	SCDFNNGITI	>10000

[a] The known, dominant epitopes are shown in bold face.
[b] Peptide binding is expressed as the IC_{50}, as described under Materials and Methods.

Immunization with vaccinia virus recombinants

Mice were immunized with vaccinia virus recombinants by ip injection of 2×10^6 PFU virus. Immunized mice were challenged with 15×10^6 PFU LCMV at least 30 days after the vaccinia virus infection.

Cr-release assays and secondary *in vitro* stimulation

Effector cells were tested for their cytolytic activity in a standard 6 h ^{51}Cr-release assay as described previously (Van der Most *et al.,* 1996). To obtain secondary CTLs, spleens from LCMV-immune mice were removed, erythrocytes were lysed by treatment with NH_4Cl, and a single-cell suspension of splenocytes was cultured for 5 days at a concentration of 4×10^6 cells/ml in the presence of 1 μg/ml free peptide in RPMI 1640 supplemented with 10% FCS, 4 mM L-glutamine, 50 mM β-mercaptoethanol, and antibiotics.

RESULTS

Identification and binding capacity of LCMV-derived D^b and K^b peptides

To identify new H-2^b-restricted LCMV epitopes, we have adapted the sequence motifs described for D^b- and K^b-restricted CTL epitopes (Falk *et al.,* 1991; Rammensee *et al.,* 1995; Saito *et al.,* 1993), to be somewhat more permissive. This was done based on the observations that differently sized peptides as well as peptides with different, yet chemically similar, C-terminal anchor residues frequently still bind MHC class I molecules with good affinity (Kast *et al.,* 1994; Kubo *et al.,* 1994; Sidney *et al.,* 1995), Thus, in the case of D^b, we have considered 10-mer peptides $N_5(MI)_{10}$, besides the canonical nonamers $N_5(MI)_9$. Also, we have utilized the motif $N_5(LV)_9$, which allows for C-terminal residues chemically similar to the standard M or I. In the case of K^b, the $(YF)_5(LM)_8$ motif was extended to also allow for V or I residues at the C-terminus. Also, the binding potential of a size variant of the canonical motif was explored by utilizing the $(YF)_5(LM)_9$ motif.

Screening of the amino acid sequences of the LCMV nucleoprotein (NP) and glycoprotein (GP) yielded 23 peptides carrying the D^b motif (Table 1) and 28 peptides containing the K^b motif (Table 2). All motif-fitting peptides were synthesized and tested for MHC class I binding using recently developed binding assays specific for the D^b and K^b class I alleles (Vitiello *et al.,* 1996). These

TABLE 2

K^b Binding of LCMV Peptides

Peptide	Sequence	Binding capacity IC_{50} (nM)[a]
GP70-77	GVYQFKSV	10
NP223-230	TSYQYLII	34
NP356-364	VGLSYSQTM	64
GP4-11	IVTMFEAL	67
NP205-212	YTVKYPNL	170
GP118-125	ISHNFCNL	371
GP45-52	ALISFLLL	933
GP256-264	KTKFFTRRL	1143
GP221-228	SQTSYQYL	1866
GP448-455	LVSIFLHL	2800
GP96-103	NSHHYISM	3111
NP121-129	ASGVYMGNL	4000
NP491-498	VWDKYGWL	>10000
GP371-378	NYSKFWYL	>10000
GP40-47	TCGIFALI	>10000
GP264-272	LAGTFTWTL	>10000
NP168-175	LNNQFGTM	>10000
NP40-47	NGLDFSEV	>10000
NP295-302	DRNPYENI	>10000
NP236-244	NISGYNFSL	>10000
NP295-303	DRNPYENIL	>10000
NP300-308	ENILYKVCL	>10000
NP418-426	QDSKYSHGM	>10000
GP311-318	HDAEFCDM	>10000
GP64-71	GPDIYKGV	>10000
GP151-158	GNSNYKAV	>10000
GP130-138	NKKTFDHTL	>10000
GP311-319	HDAEFCDML	>10000

[a] Peptide binding is expressed as the IC_{50}, as described under Materials and Methods.

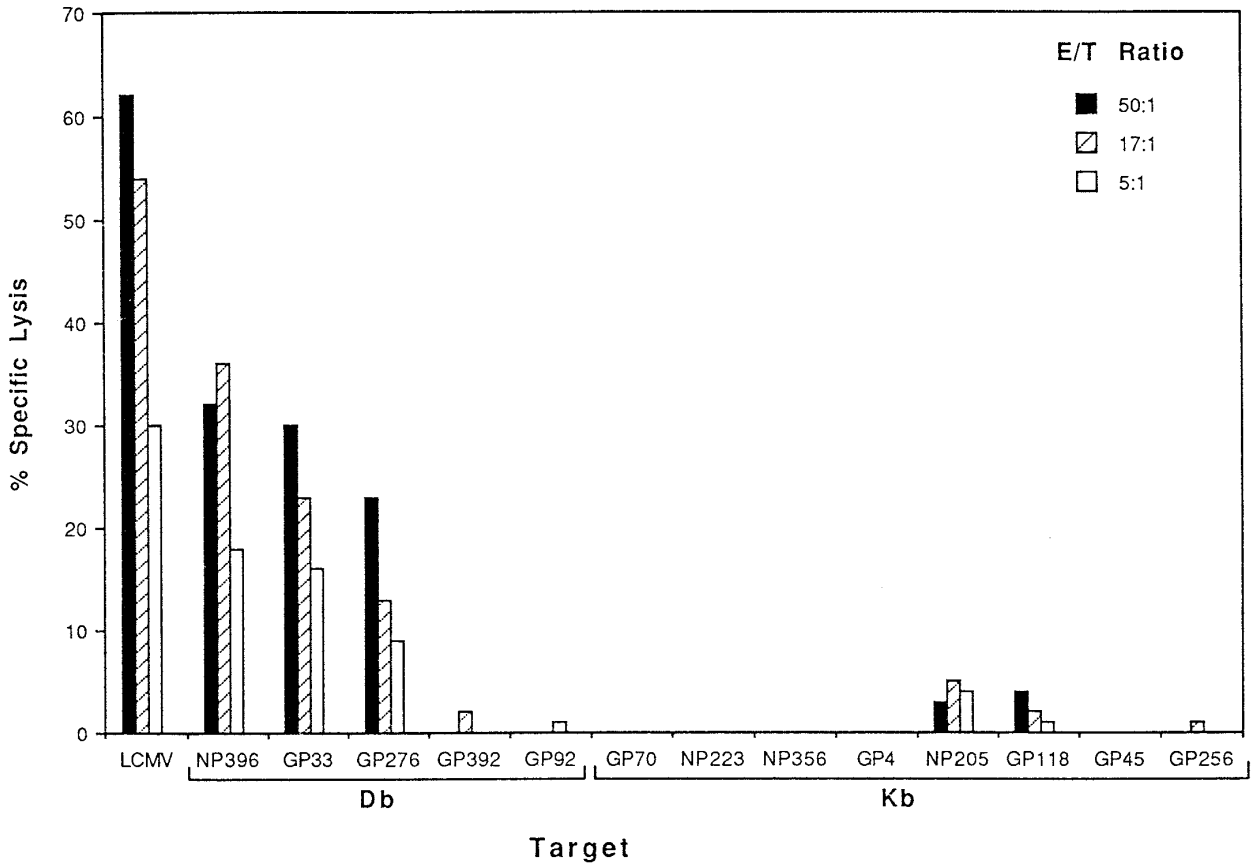

FIG. 1 Primary *ex vivo* antiviral CTL responses in C57BL/6 mice are primarily directed against the three dominant epitopes, NP396-404, GP33-43, and GP276-286. MC57 target cells were infected with LCMV clone 13 (m.o.i. = 1) or coated with the different peptides (1 μg/ml). The restriction elements of the peptides (D^b or K^b) are indicated. Specific lysis in the absence of peptide or LCMV infection has been subtracted from each value. Different effector/target (E/T) ratios are indicated.

assays are based on competition for binding of the peptide of interest with a radiolabeled standard peptide that binds the class I molecule in question with high affinity (Sette *et al.,* 1994). The concentration necessary to inhibit binding of the labeled standard peptide to solubilized class I molecules by 50%, referred to as the inhibitory concentration 50% (IC_{50} (nM), is measured and approximates the affinity (Kd) of a given peptide–MHC interaction. Sensitive D^b- and K^b-binding assays were developed using a $P_7 \rightarrow Y$ analog (SGPSNTYPEI) of the adenovirus E1A epitope (Kast *et al.,* 1989), and the VSV NP52-59 (RGYVFQGL) epitope (Van Bleek and Nathenson, 1990) as radiolabeled ligands, respectively (Vitiello *et al.,* 1996). Using these assays, IC_{50} values for each of the motif-fitting LCMV peptides and the three known epitopes were measured in two to four independent experiments. As shown in Table 2, among the three known D^b-restricted epitopes, NP396-404 was the best binder, followed by GP276-286 and GP33-41 A C-terminal extension of two amino acids, yielding GP33-43, improved MHC binding considerably, confirming earlier work by Gairin *et al.* (Gairin *et al.,* 1995). Out of the 23 new D^b motif-containing peptides, two bound MHC with intermediate affinity (IC_{50}, 50–500 nM) and two bound with low affinity (IC_{50}, 500–5000 nM) (Table 1). The remaining 19 peptides did not bind D^b. Out of 28 K^b motif-fitting peptides, we found two high-affinity binders ($IC_{50} < 50$ nM), four intermediate-affinity binders, and six low-affinity binders (Table 2). Note that a considerable number of MHC-binding peptides bears extended motifs. In fact, among the three best K^b binders, two have a noncanonical C-terminus (GP70-77 and NP223-230) and one is a nonamer (NP356-364). Furthermore, both D^b binders bear extended motifs: GP392-400 has a noncanonical C-terminus and GP92-101 is a 10-mer.

In summary, the data presented herein indicate that (including the low-affinity binders) 16 potential H-2^b-restricted CTL epitopes, in addition to the 3 known epitopes, exist in the LCMV glycoprotein and nucleoprotein sequences.

Recognition of the new MHC-binding peptides by LCMV-specific CTLs

To investigate whether any of the D^b- or K^b-binding peptides participate in the antiviral CTL response in a natural LCMV infection in mice and are in fact CTL epitopes, we studied whether these peptides are recog-

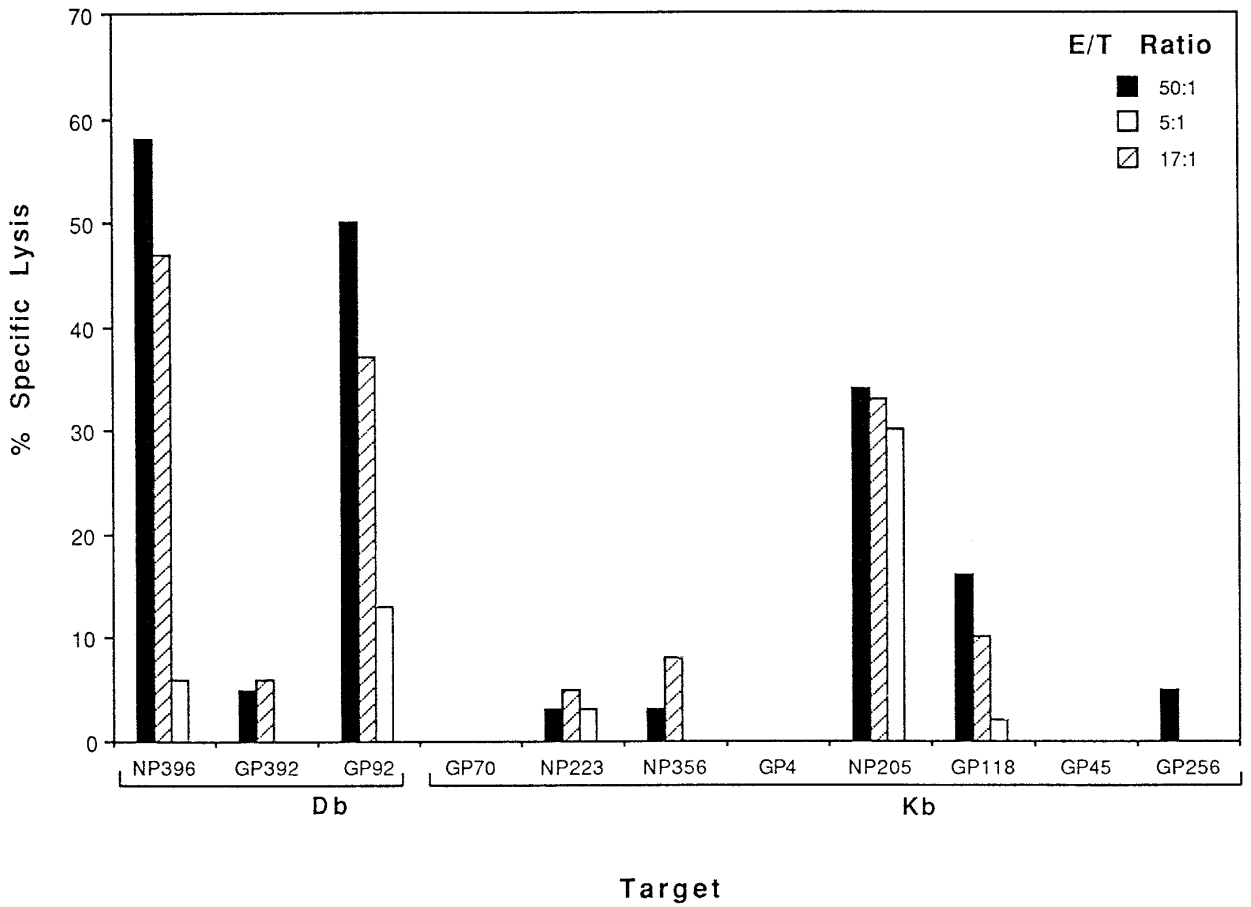

FIG. 2. Peptide specificity of secondary antiviral CTL responses in C57BL/6 mice that cleared an acute LCMV infection. Secondary CTL were obtained after *in vitro* stimulation of mouse spleen cells for 5 days with peptide (1 μg/ml). MC57 target cells were coated with the different peptides (1 μg/ml). The restriction elements of the peptides (D^b or K^b) are indicated. Specific lysis in the absence of peptide has been subtracted from each value. Different effector/target (E/T) ratios are indicated. For GP70-77, GP4-11, and GP45-52 the E/T ratios used were 25:1, 8:1, and 2.5:1.

nized by primary antiviral CTLs. In these experiments we used the two high- (GP70-77 and NP223-230) and six intermediate-affinity binders (GP392-400, GP92-101, NP356-364, GP4-11, NP205-212, and GP118-125) as well as two low-affinity binders (GP45-52 and GP256-264). To obtain primary antiviral CTLs, C57BL/6 mice ($H\text{-}2^b$) were infected with 2×10^5 PFU LCMV strain Armstrong. In adult mice, this strain causes an acute infection that is usually cleared within 8 days. Splenocytes of infected mice were harvested at eight days p.i. Next, peptide-specific CTL activity of the LCMV-primed CTLs was measured in a direct *ex vivo* ^{51}Cr-release assay on syngeneic target cells (MC57), which were coated with the different D^b- and K^b-binding peptides. As controls, CTL activities were measured on target cells coated with the dominant peptides (NP396-404, GP33-43, and GP276-286), on LCMV-infected targets, and on uncoated/uninfected targets. As shown in Fig. 1, the results clearly reveal a hierarchy among the three major epitopes. NP396-404 is immunodominant, GP33-43 is somewhat weaker, and GP276-286 is the weakest of the three dominant epitopes. However, none of the new D^b- or K^b-binding peptides (with the possible exception of NP205-212 and GP118-125) sensitized target cells for lysis, indicating that these peptides do not play a major role in the primary antiviral response. This is consistent with the notion that the antiviral response is largely directed against the NP396-404, GP33-43, and GP276-286 epitopes.

It is conceivable, however, that the dominant CTL response clears the virus so effectively that antigen levels become limiting before CTLs recognizing less-dominant epitopes have the opportunity to expand. To examine this possibility, we measured secondary CTL responses after *in vitro* stimulation of LCMV-immune splenocytes (i.e., spleen cells from mice that had previously cleared an LCMV infection) with the different D^b- and K^b-binding peptides. Specific, low-frequency CTL precursors could be amplified by this experimental approach. It is important to perform the secondary *in vitro* stimulation with peptide rather than with LCMV-infected cells, to avoid a strong bias toward the immunodominant epitopes. Accordingly, splenocytes from LCMV-immune C57/B6 mice were stimulated *in vitro* for 5 days with

TABLE 3

Vaccination with Subdominant CTL Epitopes Confers Protection against Chronic LCMV Infection

Epitope	Mouse No.	Immunization[a]	LCMV titer in serum (log_{10} PFU/ml) Day 8	Day 15
MOCK[b]	1	PBS	4.7	4.9
	2	PBS	4.7	5.2
	3	control vv[b]	4.7	4.3
	4	control vv	5.0	5.0
	5	control vv	4.8	5.0
NP396-404	1	peptide	2.2	<1.7
	2	peptide	<1.7	<1.7
	3	peptide	<1.7	<1.7
GP33-43	1	rec. vv	<1.7	<1.7
	2	rec. vv	1.7	<1.7
	3	rec. vv	<1.7	<1.7
	4	rec. vv	<1.7	<1.7
NP205-212	1	peptide	3.8	<1.7
	2	peptide	3.3	<1.7
	3	rec. vv	2.9	<1.7
	4	rec. vv	2.0	<1.7
	5	rec. vv	2.9	<1.7
	6	rec. vv	2.6	<1.7
	7	rec. vv	2.2	<1.7
GP92-101	1	peptide	2.5	<1.7
	2	peptide	3.9	2.3
	3	rec. vv	5.0	5.0
	4	rec. vv	<1.7	<1.7
	5	rec. vv	2.0	<1.7
	6	rec. vv	2.8	<1.7

[a] C57BL/6 (H-2^b) mice were immunized with peptide or with recombinant vaccinia viruses (rec. vv) as described under Materials and Methods.

[b] Mock-immunized mice were injected either with PBS (Nos. 1 and 2) or with a recombinant vaccinia virus (control vv) expressing the H-2^d-restricted LCMV epitope GP283-291 (Nos. 3–5) (van der Most *et al.,* 1996).

peptide, after which lysis of targets coated with the appropriate peptides was measured in a standard ^{51}Cr-release assay. As expected, stimulation of LCMV-immune splenocytes with the immunodominant peptide NP396-404 resulted in a strong, NP396-404-specific response (Fig. 2). However, significant peptide-specific responses were also measured after stimulation with peptides GP92-101 and NP205-212 (Fig. 2). Weak responses could be measured against GP118-125. No responses against any of the other peptides were detected. Peptide dilution experiments, in which splenocytes were stimulated with 1 μg/ml peptide and target cells were coated with serial dilutions of peptide (from 1 μg/ml peptide down to 1 pg/ml), yielded similar results, and, therefore, the same hierarchy of epitopes (data not shown). Thus, from the 10 MHC class I-binding peptides that we identified, 2 are clearly recognized during a natural LCMV infection and therefore represent 2 new LCMV-derived D^b-restricted (GP92-101) and K^b-restricted (NP205-212) CTL epitopes. GP118-125 could be an additional, K^b-restricted, subdominant epitope. Stimulation of naive splenocytes with NP205-212 or GP92-101 did not yield any peptide-specific responses (data not shown), demonstrating that the CTL activities measured after stimulation of LCMV-immune splenocytes were indeed primed by the LCMV infection. The low responses against GP92-101 and NP205-212 (as compared to the dominant epitopes, Fig. 1) indicate that these 2 peptides are subdominant epitopes.

Immunization with subdominant epitopes protects against persistent viral infection

Since 2 out of 10 MHC-binding peptides are recognized by LCMV-primed CTLs after secondary stimulation *in vitro,* we asked whether immunization with these epitopes would induce effective antiviral CTLs; i.e., are previously primed CTLs recognizing subdominant epitopes able to control a subsequent viral challenge? To evaluate epitope-specific immunity, C57BL/6 mice were immunized with peptide or with vaccinia virus recombinants expressing the subdominant epitopes as minigenes (see below). Immunized mice were subsequently challenged with 1.5×10^6 PFU LCMV clone 13. LCMV clone 13 is an Armstrong derivative that readily establishes a persistent infection in adult mice, unless antiviral memory CTL precursors are present (Lau *et al.,* 1994).

First, mice were immunized with the peptides NP396-404, NP205-212, and GP92-101 or mock-immunized with PBS. To enhance immunogenicity of the peptides, they were administrated as lipopeptide HTL–CTL constructs (Vitiello *et al.,* 1995). The lipopeptide constructs consist of the CTL epitope covalently linked to a T-helper epitope and to a lipid moiety. The T-helper epitope used in this study comprises residues 323–336 from chicken ovalbumin. Such lipopeptide constructs have been shown to be excellent immunogens using hepatitis B virus CTL epitopes (Vitiello *et al.,* 1995). Two weeks after immunization, peptide and mock-immunized mice were challenged with 1.5×10^6 PFU LCMV clone 13 and virus titers were determined 8 and 15 days later. As shown in Table 3, immunization with the dominant NP396-404 epitope conferred excellent protection: immunized mice controlled the infection within 8 days. Protection against chronic infection was also observed for the subdominant epitopes NP205-212 and GP92-101. For both peptides we found that the two immunized mice had 10- to 100-fold reduced titers at day 8 p.i. compared to those of mock-immunized mice, and had cleared the infection by day 15. None of the mock-immunized mice were able to control the infection. Thus, immunization with subdominant epitopes can confer protective immunity.

To further evaluate the potential of subdominant epitopes to induce protective immunity, vaccinia recom-

TABLE 4

Vaccination with Subdominant Epitopes Confers Protection against Chronic Infection: Tissue Titers at Day 8 Postinfection[a]

Epitope	Mouse No.	LCMV titer in tissues (log_{10} PFU/ml or g of tissue)				
		Serum	Spleen	Liver	Kidney	Lung
MOCK[b]	1	5.8	6.3	6.9	6.6	7.8
	2	5.8	6.0	6.5	6.3	8.0
	3	5.5	6.8	7.7	6.6	7.7
	4	5.5	6.3	7.3	6.0	7.0
GP33-43	1	<1.7	2.0	<1.7	3.6	2.3
	2	<1.7	<1.7	<1.7	3.0	2.0
	3	<1.7	<1.7	<1.7	3.0	2.8
	4	<1.7	<1.7	<1.7	2.0	2.6
NP205-212	1	1.7	3.8	3.0	2.8	5.0
	2	3.0	3.8	3.6	3.6	>5.0
	3	3.3	4.0	5.0	3.3	5.3
	4	3.0	4.0	4.3	3.0	5.0
GP92-101	1	3.0	3.5	4.0	3.5	4.0
	2	<1.7	3.3	<1.7	2.0	3.8
	3	<1.7	4.0	3.5	2.0	4.0

[a] C57BL/6 mice were immunized with recombinant vaccinia virus as described under Materials and Methods.

[b] Mock-immunized mice were injected with a control recombinant vaccinia virus, expressing the H-2^d-restricted LCMV epitope GP283-291 (van der Most *et al.,* 1996).

binants that expressed GP92-101 and NP205-212 as minigenes were generated (Whitton *et al.,* 1993). As a positive control, we used vaccinia virus recombinant MG34 which expresses the strong D^b-restricted epitope GP33-43 (Whitton *et al.,* 1993), and as the negative control we used a recombinant that expressed an H-2^d-restricted LCMV epitope (GP283-291) (Van der Most *et al.,* 1997, 1996). C57BL/6 mice were infected with 2×10^6 PFU recombinant vaccinia virus, and were challenged with LCMV clone 13 at least 30 days later. As shown in Table 3, mice immunized with recombinants expressing GP33-43, NP205-212, and GP92-101 rapidly cleared the virus, confirming the results of peptide immunization studies. In the case of GP33-43 (the positive control), serum virus titers were below the detection limit for three out of four mice at day 8 pi. For the two subdominant epitopes, NP205-212 and GP92-101, serum titers were 100- to 1000-fold reduced as compared to those of the negative control at day 8 p.i. and were below detection levels at day 15. Analysis of virus titers in different organs at day 8 p.i. indicated that mice immunized with subdominant epitopes clear the infection with somewhat slower kinetics than MG34 immunized mice (Table 4). Serum titers in these mice were low and there was also a 10- to 1000-fold drop in virus levels in the different tissues.

DISCUSSION

In the present study, we have identified two new H-2^b-restricted, subdominant LCMV epitopes, i.e., the D^b-restricted epitope GP92-101 and the K^b-restricted epitope NP205-212. Both peptides bind their restricting class I molecules with intermediate affinity (IC_{50} values are 470 and 170 nM, respectively), induce protective immunity, and are recognized by LCMV-primed CTLs after secondary *in vitro* stimulation of memory T-cells with peptide. The K^b-binding peptide GP118-125 could be an additional subdominant epitope, since weak responses against this peptide could be detected. Our data also reveal a clear hierarchy in antiviral responses in H-2^b mice. In a direct *ex vivo* cytotoxicity assay, NP396-404 is the immunodominant epitope, followed by GP33-43 and GP276-286.

The identification of three (including GP118-125) new subdominant CTL epitopes illustrates the value of combining motif searches with MHC class I-binding assays. As summarized in Table 5, analysis of 51 D^b/K^b motif-fitting peptides yielded two high-, six intermediate, and eight low-affinity binders. Out of these 16 peptides, 3 were new CTL epitopes. In a previous study, we analyzed 34 D^d/K^d motif-fitting peptides, and found one high-, four intermediate-, and five low-affinity binders; 2 of the 10 peptides were new CTL epitopes (Table 5) (Van der Most *et al.,* 1996). Thus, by combining motif searches with MHC class I-binding assays new subdominant epitopes can be readily identified, although a considerable number of peptides has to be tested. Strikingly, all new subdominant epitopes are intermediate binders (Table 5), indicating that an IC_{50} value lower than 500 nM is critical (Sette *et al.,* 1994). On the other hand, high-affinity binders tend to be either dominant epitopes, e.g., NP396-404 (IC_{50}, 4.4 nM) and the L^d-restricted immunodominant LCMV epitope NP118-126 (IC_{50}, 13 nM) (Van der Most *et al.,* 1996), or no epitopes at all, e.g., K^b binders GP70-77

TABLE 5

Motif-Fitting Peptides in the LCMV NP and GP[a]

MHC class I allele	Total number of motif-fitting peptides tested	Number of binders[b]		
		High $IC_{50} < 50$ nM	Intermediate IC_{50} 50–500	Low IC_{50} 500–5000
D^b	23 (1)[c]	0 (0)	2 (1)	2 (0)
K^b	28 (1)	2 (0)	4 (2)	6 (0)
D^d	18 (0)	0 (0)	0 (0)	3 (0)
K^d	16 (2)	1 (0)	4 (2)	2 (0)

[a] Motif-fitting peptides are shown for class I alleles K^b/D^b (this study) and K^d/D^d (van der Most *et al.,* 1996).

[b] Peptides are distributed among high-, intermediate-, and low-affinity binders.

[c] Numbers in parentheses indicate CTL epitopes during LCMV infection. Note that all of the newly identified subdominant epitopes are intermediate binders and that none of the high-affinity binders are new epitopes. In contrast, the known, immunodominant epitopes tend to be high-affinity binders, suggesting that high-affinity binders are either dominant epitopes or not epitopes at all (see Discussion).

and NP223-230 (IC_{50}, 10 and 34 nM, respectively) and the K^d-binding LCMV peptide NP314-322 (IC_{50}, 4.8 nM) (Van der Most *et al.,* 1996). Thus, subdominant epitopes are most likely to be found among intermediate binders, whereas the absence of epitopes among the new high-affinity binders, GP70-77, NP223-230, and NP314-322 (Van der Most *et al.,* 1996), probably reflects a hole in the TCR repertoire or a processing problem.

Using a similar approach as described here, Oldstone and co-workers identified 34 NP and GP peptides that fit the D^b—MHC class I-binding motif (Hudrisier *et al.,* 1996; Oldstone *et al.,* 1995). They found that 3 of these peptides, including GP92-101, bound D^b. However, they were not able to detect LCMV-specific CTL that recognized GP92-101 and concluded that this viral peptide was not a CTL epitope. The most likely explanation for this result is the secondary CTL protocol used by these investigators in which spleen cells from LCMV-infected mice were restimulated with virus-infected cells for 5 days and then tested for cytolytic activity on peptide-coated targets. Such conditions greatly favor the expansion of CTL recognizing dominant epitopes and it is very easy to miss subdominant responses after restimulation with virus-infected cells. As pointed out previously, in order to detect subdominant CTL responses, it is critical to restimulate with the specific peptide (Van der Most *et al.,* 1996). The precursor frequency of GP92-101-specific CTL in LCMV immune mice is 10- to 100-fold lower than the precursor frequency of CTL specific for the dominant epitopes (K. Murali-Krishna and R. Ahmed, unpublished data) and therefore selective stimulation (i.e., with peptide) is necessary to detect the subdominant GP92-101 response. A possible concern regarding CTL obtained by peptide restimulation is whether these peptide-activated CTL are "truly" virus specific and can recognize virus-infected cells. In this study, we have shown that this is indeed the case: not only do GP92-101-stimulated CTL kill LCMV-infected targets, but, even more importantly, vaccination with the GP92-101 epitope confers protective immunity.

The fact that these epitopes do elicit protective immunity makes them excellent candidates for therapeutic immunization studies. Chronic LCMV infection in mice is thought to result from the "exhaustion" (deletion) of LCMV-specific CTLs following overwhelming antigenic stimulation (Hotchin, 1971; Moskophides *et al.,* 1993). It is conceivable that CTLs recognizing subdominant epitopes such as GP92-101 and NP205-212 may escape deletion and could be activated once a chronic infection is established. Also, variants of these peptides with different anchor residues could be explored for their use as therapeutic vaccines. The potential of using epitope analogs is illustrated in two recent studies, in which melanoma-specific (Bakker *et al.,* 1997) and influenza A-specific CTL epitopes were modified (Tourdot *et al.,* 1997). In both cases, replacements of the anchor residues resulted in better MHC class I binding. In addition, immunization with modified subdominant influenza A epitopes induced strong protective immunity (Tourdot *et al.,* 1997). Thus, increasing MHC class I binding of the LCMV subdominant epitopes described here and previously (Van der Most *et al.,* 1997, 1996) may increase the immunogenicity of these epitopes and turn them into excellent candidates for therapeutic vaccination studies.

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